BEST AVAILABLE COPY

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
23 October 2003 (23.10.2003)

PCT

(10) International Publication Number
WO 03/086456 A2

(51) International Patent Classification[7]: A61K 39/395, C07K 16/30, C12N 5/20, G01N 33/574, A61P 35/00

(21) International Application Number: PCT/CA03/00500

(22) International Filing Date: 7 April 2003 (07.04.2003)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/370,659 5 April 2002 (05.04.2002) US

(71) Applicant *(for all designated States except US)*: **ARIUS RESEARCH, INC.** [CA/CA]; 55 York Street, 16th Floor, Toronto, Ontario M5J 1R7 (CA).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **YOUNG, David** [CA/CA]; 33 University Avenue, #2407, Toronto, Ontario M5J 1L1 (CA). **DUAN, Xiaobo** [CA/CA]; 116 Muirland Cres., Brampton, Ontario L6X 4G2 (CA). **GROVES, Timothy, C.** [CA/CA]; 3319 Dundas St. W, #4, Toronto, Ontario M6P 2A6 (CA).

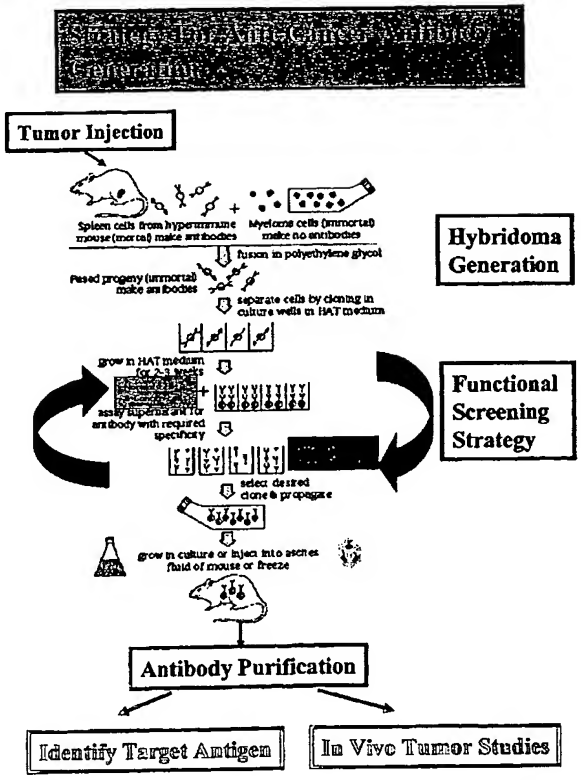
(74) Agent: **SINGLEHURST, John, C.**; Finlayson & Singlehurst, 70 Gloucester Street, Ottawa, Ontario K2P 0A2 (CA).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE,

[Continued on next page]

(54) Title: ANTIGENIC PROFILING OF NEOPLASTIC CELLS, ONCOGENIC THERAPY UTILIZING FUNCTIONAL ANTIBODIES THERETO AND CYTOTOXIC IMMUNE COMPLEXES FORMED THEREBY



(57) Abstract: The present invention relates to a method for producing patient specific anti-cancer antibodies using a novel paradigm of screening and characterizing the target antigen thereof. The invention further relates to the process by which the antibodies are made and to their methods of use. The antibodies can be used in aid of staging and diagnosis of a cancer, and can be used to treat primary tumors and their metastases. The anti-cancer antibodies can be combined with a variety of anti-neoplastic agents, e.g. Cisplatin, to derive a synergistic effect with regard to reduction in tumour growth kinetics and metastasis.

WO 03/086456 A2

ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, RO, SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:

— *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii)) for the following designations AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, UZ, VC, VN, YU, ZA, ZM, ZW, ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, RO, SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG)*

— *as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii)) for all designations*

— *of inventorship (Rule 4.17(iv)) for US only*

Published:

— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

ANTIGENIC PROFILING OF NEOPLASTIC CELLS, ONCOGENIC THERAPY UTILIZING FUNCTIONAL ANTIBODIES THERETO AND CYTOTOXIC IMMUNE COMPLEXES FORMED THEREBY

Reference to Related Applications:

This application relates to S.N. 09/727,361, filed November 29, 2000, and U.S. Patent 6,180,357, the contents of which are herein incorporated by reference.

Field of the Invention:

This application is directed toward the use of functional antibodies having the ability to bind to an epitope of Cytokeratin 18 (CK18) either alone or as an obligate heterodimer in combination with Cytokeratin 8 (CK8); to detection, localization and treatment of neoplastic cells whose cell membrane possesses an antigenic profile embodying the required binding site necessary for formation of a cytotoxic immune complex upon binding with said functional monoclonal antibodies, and to oncogenic therapy of said cells via utilization of said functional antibodies either alone or in combination with traditional neoplastic agents.

Background of the Invention:

Each individual who presents with cancer is unique and has a cancer that is as different from other cancers as that person=s identity. Despite this, current therapy treats all patients with the same type of cancer, at the same stage, in the same way. At least 30% of these patients will fail the first line therapy, thus leading to further rounds of treatment and the increased probability of treatment failure, metastases, and ultimately, death. A superior approach to treatment would be the customization of therapy for the particular individual. The only current therapy which lends itself to customization is surgery. Chemotherapy and radiation treatment can not be tailored to the patient, and surgery

by itself, in most cases is inadequate for producing cures.

The development of anti-cancer monoclonal antibodies (mAbs) has raised the possibility of tailoring them for personalized therapy based on the antigenic profile of the patient=s tumor. By making use of a method for producing functional anti-cancer mAbs through the use of patient biopsy samples and a novel paradigm of screening (in accordance with the teachings of U.S. 6,180,357), a library of anti-cancer mAbs may be generated that can be used alone, in combination with other mAbs based on the expression of antigens on the patient=s tumor, or furthermore in combination with traditional neoplastic agents in the hope of deriving an additive or possibly even a synergistic effect.

Having recognized that a significant difference between cancerous and normal cells is that cancerous cells contain antigens that are specific to transformed cells, the scientific community has long held that monoclonal antibodies can be designed to specifically target transformed cells by binding specifically to these cancer antigens; thus giving rise to the belief that monoclonal antibodies can serve as "Magic Bullets" to eliminate cancer cells.

Prior Art:

U.S. Patent No. 5,750,102 discloses a process wherein cells from a patient's tumor are transfected with MHC genes which may be cloned from cells or tissue from the patient. These transfected cells are then used to vaccinate the patient.

U.S. Patent No. 4,861,581 discloses a process comprising the steps of obtaining monoclonal antibodies that are specific to an internal cellular component of neoplastic and normal cells of the mammal but not to external components, labeling the monoclonal antibody,

contacting the labeled antibody with tissue of a mammal that has received therapy to kill neoplastic cells, and determining the effectiveness of therapy by measuring the binding of the labeled antibody to the internal cellular component of the degenerating neoplastic cells. In preparing antibodies directed to human intracellular antigens, the patentee recognizes that malignant cells represent a convenient source of such antigens.

U.S. Patent No. 5,171,665 provides a novel antibody and method for its production. Specifically, the patent teaches formation of a monoclonal antibody which has the property of binding strongly to a protein antigen associated with human tumors, e.g. those of the colon and lung, while binding to normal cells to a much lesser degree.

U.S. Patent No. 5,484,596 provides a method of cancer therapy comprising surgically removing tumor tissue from a human cancer patient, treating the tumor tissue to obtain tumor cells, irradiating the tumor cells to be viable but non-tumorigenic, and using these cells to prepare a vaccine for the patient capable of inhibiting recurrence of the primary tumor while simultaneously inhibiting metastases. The patent teaches the development of monoclonal antibodies which are reactive with surface antigens of tumor cells. As set forth at col. 4, lines 45 et seq., the patentees utilize autochthonous tumor cells in the development of monoclonal antibodies expressing active specific immunotherapy in human neoplasia.

U.S. Patent No. 5,725,856 is directed toward treatment of carcinomas which overexpress HER2 receptor comprising administration of an antibody which binds to the extracellular domain of the HER2 receptor, thereby reducing or eliminating a patient=s tumor burden. The patent teaches conjugation of the antibody to a cytotoxic moiety.

U.S. Patent No. 5,783,186 is drawn to Anti-Her2 antibodies which induce apoptosis in Her2 expressing cells, hybridoma cell lines producing the antibodies, methods of treating cancer using the antibodies and pharmaceutical compositions including said antibodies.

U.S. Patent No. 5,849,876 describes new hybridoma cell lines for the production of monoclonal antibodies to mucin antigens purified from tumor and non-tumor tissue sources.

U.S. Patent No. 5,869,045 relates to antibodies, antibody fragments, antibody conjugates and single chain immunotoxins reactive with human carcinoma cells. The mechanism by which these antibodies function is two-fold, in that the molecules are reactive with cell membrane antigens present on the surface of human carcinomas, and further in that the antibodies have the ability to internalize within the carcinoma cells, subsequent to binding, making them especially useful for forming antibody-drug and antibody-toxin conjugates. In their unmodified form the antibodies also manifest cytotoxic properties at specific concentrations.

U.S. Patent No. 6,207,153 is directed towards antigen binding fragments recognized by H11, described as the C-antigen, nucleotides encoding the fragments, and their use for prophylaxis and detection of cancers.

Eto et al, AMapping and Regulation of the Tumor-associated Epitope Recognized by Monoclonal Antibody RS-11 (Journal of Biological Chemistry, Vol. 275, No. 35, 9/2000, Pp. 27075-27083) discloses an antibody recognized by a tumor-associated antigen. The RS-11 antibody appears to recognize an epitope of Keratin 18 and/or Keratin 8 expressed in neoplastic cells, but not present in normal cells. In contrast, the antibody of the instant invention, ARH460-23, appears to bind with an epitope of CK18 within the cytoplasm or perinuclear region of normal

cells, while only recognizing a binding site residing upon the cellular membrane of neoplastic cells.

Oshima et al, AOncogenic Regulation and Function of Keratins 8 and 18" (Cancer and Metastasis Reviews 15:445-471, 1996, disclose the widespread expression of CK8 and CK18 in carcinoma cells, and indicate that they are a useful tool in the understanding of cancer and metastasis.

Summary of the Invention:

A variety of therapeutic anti-cancer monoclonal antibodies (mAbs) are commonly generated involving a strategy whereby mAbs are formed against a single target and screened for their ability to bind a well-defined tumor-associated antigen. Furthermore it is known to generate functional therapeutic anti-cancer mAbs by using tumor cells as immunogens to provide an extensive array of target antigens and to screen for anti-cancer activity instead of binding. The present invention has utilized a method in accordance with U. S. Patent No. 6,180,357, whereby anti-cancer mAbs are produced through the use of patient biopsy samples and a novel model of screening that selects for antibody-producing clones that discriminately kill tumor cells and not normal cells. This strategy was used to generate a plurality of mAbs, from which a particular antibody designated ARH460-23, a murine IgM, kappa mAb that was generated in response to a lung tumor biopsy, was selected for further study. The binding activity of ARH460-23 is characterized in accordance with the instant invention and the in vivo efficacy of ARH460-23 as an anti-tumor agent, having a particular activity against the lung cancer line, NCI H460 is demonstrated. The ARH460-23 antigen is identified as the well-defined tumor-associated antigen, cytokeratin 18. The methodology employed herein creates an opportunity for generating

functional mAbs towards tumor-associated antigens using tumor tissue, instead of a defined target.

The anti-cancer antibodies of the instant invention may be used alone, in combination with other mAbs based on the expression of antigens on the patient=s tumor, or alternatively in combination with other neoplastic agents, for example radioisotopes, vinca alkaloids, adriamycin, bleomycin sulfate, Carboplatin, Cisplatin, cyclophosphamide, Cytarabine, Dacarbazine, Dactinomycin, Duanorubicin hydrochloride, Doxorubicin hydrochloride, Etoposide, fluorouracil, lomustine, Mechlororethamine hydrochloride, melphalan, mercaptopurine, methotrexate, mitomycin, mitotane, pentostatin, pipobroman procarbaze hydrochloride, streptozotocin, taxol, thioguanine, Uracil mustard, and the like.

Antibodies produced in the context of the present invention, were generated in mice immunized with fixed cells from one of several types of tumor biopsies. Functional mAbs were identified using a selective screening process and evaluated for in vitro cytotoxicity towards tumor cells, in vivo anti-tumor activity and tissue specificity. Investigational techniques were utilized to determine the formation of immune complexes and the antigenic target of the antibody was thereby determined.

Within the context of this application, anti-cancer antibodies having either cell-killing (cytotoxic) or cell-growth inhibiting (cytostatic) properties will hereafter be referred to as cytotoxic. These antibodies can be used in aid of staging and diagnosis of a cancer, and can be used to treat tumor metastases. The antibodies may be conjugated to other hematogenous cells, e.g. lymphocytes, macrophages, monocytes, natural killer cells, etc.

Accordingly, it is an objective of the invention to

teach one or more novel anti-cancer antibodies which are cytotoxic with respect to cancer cells while simultaneously being relatively non-toxic to non-cancerous cells.

It is an additional objective of the invention to identify and characterize the antigenic target for the anti-cancer antibody.

It is yet an additional objective of the instant to determine the point of formation, at a sub-cellular level, of the immune complex, wherein said point of formation evidences an oncogenic variation in the cell.

A still further objective of the instant invention is to produce anti-cancer antibodies which are useful for diagnosis, prognosis, localization, and signaling of oncogenic change at a cellular level.

It is yet an additional objective of the instant invention to treat patients having primary cancers which express CK18 and metastatic tumors thereof.

Brief Description of the Figures

Figure 1 is an outline of a strategy for Anti-Cancer Antibody generation, purification, and target antigen identification;

Figure 2 describes metastasis reduction in an orthotopic implantation model;

Figure 3 is a 2-D gel analysis of NCI H460 membrane proteins probed with ARH460-23;

Figure 4 is a 2-D gel analysis of membrane proteins from Jurkat cells probed with ARH460-23;

Figure 5 is a graphical representation of flow cytometric analysis showing differential reactivity of ARH460-23 for Jurkat and NCI H460 cells;

Figure 6 portrays the effect of preventative therapy on tumour growth kinetics utilizing a combination of antibody and anti-neoplastic agent;

Figure 7 is a tabular analysis of an immunohistochemistry study carried out on formalin-fixed, paraffin-embedded human tissues to profile expression of the ARH460-23 antigen on normal and tumor tissues;

Figure 8 represents the result of immunohistochemical staining of ARH460-23 with NCI H460 and Jurkat cell pellets.

Detailed Description of the Invention:

It is to be understood that while a certain form of the invention is illustrated, it is not to be limited to the specific form or arrangement herein described and shown. It will be apparent to those skilled in the art that various changes may be made without departing from the scope of the invention and the invention is not to be considered limited to what is shown and described in the specification.

Other objects and advantages of this invention will become apparent from the following description wherein are set forth, by way of illustration and example, certain embodiments of this invention.

Traditionally, monoclonal antibodies have been made according to fundamental principles laid down by Kohler and Milstein. Mice are immunized with antigens, with or without, adjuvants. The splenocytes are harvested from the spleen for fusion with immortalized hybridoma partners. These are seeded into microtitre plates where they can secrete antibodies into the supernatant that is used for cell culture. To select from the hybridomas that have been plated for the ones that produce antibodies of interest the hybridoma supernatants are usually tested for antibody binding to antigens in an ELISA (enzyme linked immunosorbent assay) assay. The idea is that the wells that contain the hybridoma of interest will contain antibodies that will bind most avidly to the test antigen, usually the immunizing antigen. These wells are then

subcloned in limiting dilution fashion to produce monoclonal hybridomas. The selection for the clones of interest is repeated using an ELISA assay to test for antibody binding. Therefore, the principle that has been propagated is that in the production of monoclonal antibodies the hybridomas that produce the most avidly binding antibodies are the ones that are selected from among all the hybridomas that were initially produced. That is to say, the preferred antibody is the one with highest affinity for the antigen of interest.

There have been many modifications of this procedure such as using whole cells for immunization. In this method, instead of using purified antigens, entire cells are used for immunization. Another modification is the use of cellular ELISA for screening. In this method instead of using purified antigens as the target in the ELISA, fixed cells are used. In addition to ELISA tests, complement mediated cytotoxicity assays have also been used in the screening process. However, antibody-binding assays were used in conjunction with cytotoxicity tests. Thus, despite many modifications, the process of producing monoclonal antibodies relies on antibody binding to the test antigen as an endpoint.

Most antibodies directed against cancer cells have been produced using the traditional methods outlined above. These antibodies have been used both therapeutically and diagnostically. In general, for both these applications, the antibody has been used as the targeting agent that delivers a payload to the site of the cancer. These antibody conjugates can either be radioactive, toxic, or serve as an intermediary for further delivery of a drug to the body, such as an enzyme or biotin. Furthermore, it was widely held, until recently, that naked antibodies had little effect *in vivo*. Both HERCEPTIN and RITUXIMAB are humanized murine

killing need not be predicated upon screening of the hybridomas for the best binding antibodies. Rather, although not advocated by those who produce monoclonal antibodies, the screening of the hybridoma supernatants for cell killing or alternatively for cessation of growth of the cancerous cells may be selected as a desirable endpoint for the production of cytotoxic or cytostatic antibodies. It is well understood that the *in-vivo* antibodies mediate their function through the Fc portions and that the utility of the therapeutic antibody is determined by the functionality of the constant region or attached moieties. In this case the FAb portion of the antibody, the antigen-combining portion, will confer to the antibody its specificity and the Fc portion its functionality. The antigen combining site of the antibody can be considered to be the product of a natural combinatorial library. The result of the rearrangement of the variable region of the antibody can be considered a molecular combinatorial library where the output is a peptide. Therefore, the sampling of this combinatorial library can be based on any parameter. Like sampling a natural compound library for antibiotics, it is possible to sample an antibody library for cytotoxic or cytostatic compounds.

The various endpoints in a screen must be differentiated from each other. For example, the difference between antibody binding to the cell is distinct from cell killing. Cell killing (cytotoxicity) is distinct from the mechanisms of cell death such as oncosis or apoptosis. There can be many processes by which cell death is achieved and some of these can lead either to oncosis or apoptosis. There is speculation that there are other cell death mechanisms other than oncosis or apoptosis but regardless of how the cell arrives at death there are some commonalities of cell death. One of these

monoclonal antibodies that have recently been approved for human use by the FDA. However, both these antibodies were initially made by assaying for antibody binding and their direct cytotoxicity was not the primary goal during the production of hybridomas. Any tendency for these antibodies to produce tumor cell killing is thus through chance, not by design.

Although the production of monoclonal antibodies have been carried out using whole cell immunization for various applications the screening of these hybridomas have relied on either putative or identified target antigens or on the selectivity of these hybridomas for specific tissues. It is axiomatic that the best antibodies are the ones with the highest binding constants. This concept originated from the basic biochemical principle that enzymes with the highest binding constants were the ones that were the most effective for catalyzing a reaction. This concept is applicable to receptor ligand binding where the drug molecule binding to the receptor with the greatest affinity usually has the highest probability for initiating or inhibiting a signal. However, this may not always be the case since it is possible that in certain situations there may be cases where the initiation or inhibition of a signal may be mediated through non-receptor binding. The information conveyed by a conformational change induced by ligand binding can have many consequences such as a signal transduction, endocytosis, among the others. The ability to produce a conformational change in a receptor molecule may not necessarily be due to the filling of a ligand receptor pocket but may occur through the binding of another extra cellular domain or due to receptor clustering induced by a multivalent ligand.

As disclosed in U.S. Patent 6,180,357, and outlined in Figure 1, the production of antibodies to produce cell

is the absence of metabolism and another is the denaturation of enzymes. In either case vital stains will fail to stain these cells. These endpoints of cell death have been long understood and predate the current understanding of the mechanisms of cell death. Furthermore, there is the distinction between cytotoxic effects where cells are killed and cytostatic effects where the proliferation of cells are inhibited.

In a preferred embodiment of the present invention, the assay is conducted by focusing on cytotoxic activity toward cancerous cells as an end point. In a preferred embodiment, a live /dead assay kit , for example the LIVE/DEAD[7] Viability/Cytotoxicity Assay Kit (L-3224) by Molecular Probes, is utilized. The Molecular Probes kit provides a two-color fluorescence cell viability assay that is based on the simultaneous determination of live and dead cells with two probes that measure two recognized parameters of cell viability C intracellular esterase activity and plasma membrane integrity. The assay principles are general and applicable to most eukaryotic cell types, including adherent cells and certain tissues, but not to bacteria or yeast. This fluorescence-based method of assessing cell viability is preferred in place of such assays as trypan blue exclusion, Cr release and similar methods for determining cell viability and cytotoxicity.

In carrying out the assay, live cells are distinguished by the presence of ubiquitous intracellular esterase activity, determined by the enzymatic conversion of the virtually nonfluorescent cell-permeant CALCEIN AM to the intensely fluorescent Calcein. The polyanionic dye Calcein is well retained within live cells, producing an intense uniform green fluorescence in live cells (ex/em ~495 nm/~515 nm). EthD-1 enters cells with damaged membranes and undergoes a 40-fold enhancement of

fluorescence upon binding to nucleic acids, thereby producing a bright red fluorescence in dead cells (ex/em ~495 nm/~635 nm). EthD-1 is excluded by the intact plasma membrane of live cells. The determination of cell viability depends on these physical and biochemical properties of cells. Cytotoxic events that do not affect thèse cell properties may not be accurately assessed using this method. Background fluorescence levels are inherently low with this assay technique because the dyes are virtually nonfluorescent before interacting with cells.

The antibodies are designed and can be used for therapeutic treatment of cancer in patients. Ideally the antibodies can be naked antibodies. They can also be conjugated to toxins. They can be used to target other molecules to the cancer. e.g. biotin conjugated enzymes. Radioactive compounds can also be used for conjugation.

The antibodies can be fragmented and rearranged molecularly. For example Fv fragments can be made; sFv-single chain Fv fragments; diabodies etc.

It is envisioned that these antibodies can be used for diagnosis, prognosis, localization and monitoring of cancer and oncogenic changes at a cellular or sub-cellular level. For example the patients can have blood samples drawn for shed tumor antigens which can be detected by these antibodies in different formats such as ELISA assays, rapid test panel formats etc. The antibodies can be used to stain tumor biopsies for the purposes of diagnosis. In addition a panel of therapeutic antibodies can be used to test patient samples to determine if there are any suitable antibodies for therapeutic use.

In isolation of ARH460-23 customized anti-cancer antibodies were produced to a patient-s lung cancer cells, but cultured cells were used in the antibody development process to demonstrate the generality of the immunization process. The samples were prepared into single cell

suspensions and fixed for injection into mice. After the completion of three rounds of immunization with cells derived directly from a patient=s lung cancer, the mice were immunized twice with a human lung large cell carcinoma cell line (NCI-H460). Hybridomas were produced from splenocytes and the supernatants were screened against a variety of cancer cell lines and normal cells in standard cytotoxicity assays. Those hybridomas that were reactive against cancer cell lines but were not reactive against normal non-transformed cells were selected for further propagation. Clones that were considered positive were ones that selectively killed the cancer cells but did not kill the non-transformed cells. The antibodies are characterized for a large number of biochemical parameters and then humanized for therapeutic use.

The lung tumor cells isolated and cell lines were cultured. Balb/c mice, A strain with $H\text{-}2^d$ haplotype from Charles River Canada, St. Constant, Quebec, Canada, female, 7-8 week old, were immunized with the human lung cancer cells emulsified in an equal volume of either Freund's complete adjuvant (FCA) for the first immunization and then in Freund's incomplete adjuvant (FIA) for subsequent immunizations at 0, 21, 45 days with $5x10^5$ cells. The mice were immunized with fixed NCI H460 cells, which were prepared from NCI H460 cells grown in T-75 cell culture flask by scraping mono-layer cells into cell suspensions at 105, 150 and 170 days. Immunized mice were sacrificed 3-4 days after the final immunization with NCI H460 cells, given intra-peritoneally, in phosphate buffered saline buffer (PBS), pH 7.4. The spleens were harvested and the splenocytes were divided into two aliquots for fusion with Sp2/0 myeloma partners using the methods outlined in Example 1.

The screening was carried out 10 days after the fusion against NCI H460 cells and CCD-27SK. Antibodies were

characterized for binding to different cell lines with a cellular ELISA.

The wells that were considered positive were subcloned and the same screening process was repeated 9 days and 18 days later. A number of monoclonal antibodies were produced in accordance with the method of the present invention, including that which produces the antibody designated ARH460-23. The ARH460-23 antibody is produced by a hybridoma cell line deposited on November 21, 2000 with the American Type Culture Collection at 10801 University Boulevard, Manassas, Va. having an ATCC Accession Number PTA-2700. Upon issuance of a patent, access to this cell line will not be withheld.

Clones were able to produce antibodies that had a greater than 15% killing rate for cancerous cells and at the same time some of the clones were able to produce less than eight percent killing of normal control fibroblasts.

The anti-cancer antibodies of the invention are useful for treating a patient with a cancerous disease when administered in admixture with a pharmaceutically acceptable adjuvant, for example normal saline, a lipid emulsion, albumen, phosphate buffered saline or the like and are administered in an amount effective to mediate treatment of said cancerous disease, for example with a range of about 1 microgram per milliliter to about 1 gram per milliliter.

The method for treating a patient suffering from a cancerous disease may further include the use of conjugated anti-cancer antibodies and would thus include conjugating patient specific anti-cancer antibodies with a member selected from the group consisting of toxins, enzymes, radioactive compounds, and hematogenous cells; and administering these conjugated antibodies to the patient; wherein said anti-cancer antibodies are administered in admixture with a pharmaceutically acceptable adjuvant, for example normal saline, a lipid emulsion, albumen, phosphate buffered saline or the like and are administered in an amount

effective to mediate treatment of said cancerous disease, for example with a range of about 1 microgram per mil to about 1 gram per mil. In a particular embodiment, the anti-cancer antibodies useful in either of the above outlined methods may be a humanized antibody.

An experiment to determine the effects of antibody administration in conjunction with anti-neoplastic agents ws carried out and is summarized as follows:

MATERIALS AND METHODS

In Vivo Study Protocols

Heterotopic Tumour Cell Engraftment

For all animals, $1x10^6$ cells (100 CL of PBS containing $1x10^7$ cell/mL) of the lung cancer cell line NCI-H460 were implanted subcutaneously into the scruff of the neck of seven week old female SCID mice.

Treatment

The study consisted of 4 groups of 10 mice. Treatment was initiated 3 days after tumor cell engraftment. Group 1 received the chemotherapeutic drug cisplatin (3.5 mg/kg) on treatment days 1, 5 and 9. Group 2 received injections of 25 mg/kg of antibody ARH460-23 three times a week for period of three weeks. Group 3 received a combination of cisplatin (3.5 mg/kg) on day 1, 5 and 11 as well as ARH460-23 (25 mg/kg) three times a week for a total of 3 weeks. The control group (Group 4) received 500 CL of 0.9% normal saline three times a week for a total of three weeks.

Study Animal Observation and Endpoints

Animals were handled under in accordance with prescribed practices as outlined by the Canadian Council on Animal Care (CCAC). All mice were weighed and examined three times a week for clinical signs of toxicity such as ruffled fur, lethargy or skin lesions. Once the tumors grew to a size that was palpable, measurements of tumor length (a) and width (b) were made with calipers and recorded. Tumor volume was calculated using the formula: V (tumor volume)= $ab^2/2$. Tumor bearing animals were euthanized by CO_2 overdose when the tumor mass compromised normal behavior/physiology, suffered severe weight loss or tumors ulcerated according to the CCAC and University Health Network guidance document on experimental endpoints.

Orthotopic Tumour Engraftment

Green fluorescent protein (GFP)-labelled NCI H460 cells were injected subcutaneously into five to six week old female NCr-nu mice to generate stock tumor tissue. When the tumors grew to log phase, they were harvested and cut into small fragments of 1 mm^3 each. One fragment of tumor tissue was then surgically implanted orthotopically into 5-6 week old female NCr-nu mice.

Treatment

The study consisted of 4 groups of 10 mice. Group 1 received the chemotherapeutic drug cisplatin (3.5 mg/kg) on treatment days 1, 5 and 9. Group 2 received injections of 25 mg/kg of antibody ARH460-23 three times a week for period of three weeks. Group 3 received a combination of cisplatin (3.5 mg/kg) on day 1, 5 and 11 as well as ARH460-23 (25 mg/kg) three times a week for a total of 3 weeks. The control

group (Group 4) received normal saline three times a week for a total of three weeks.

Study Animal Observation and Endpoints

Animal studies were conducted in accordance with the principles and procedures outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals. At the end of the study (day 42 post implantation), tumor measurement of GFP imaging was determined by opening the thoracic wall. The primary tumors were excised and weighed at the end of the study.

In a xenograft tumor model of lung cancer, ARH460-23 has adjuvant potential when used in combination with cisplatin. Combination therapy of both ARH460-23 and cisplatin significantly reduced tumor volume in SCID mice injected subcutaneously with NCI H460 cells. The combination treatment also caused a trend towards disease-free survival.

In a xenograft tumor model of lung cancer metastasis, ARH460-23 significantly inhibited metastasis of NCI H460 tumor tissue following orthotopic transplantation. ARH460-23 significantly reduced contralateral lung and thoracic lymph nodes metastasis of GFP-labelled NCI H460 tissue as well as primary tumor size. This work was performed in collaboration with Anti-Cancer Inc., San Diego, CA.

ARH460-23 Target Antigen

Keratin 18 (K18), a type I keratin, is a member of the intermediate filament protein family that exists as an obligate heterodimer with Keratin 8 (K8), a type II keratin. The gene was cloned in 1986 and its expression in normal and cancer tissues has been studied extensively. K18 and K8 form alpha helical coil-coil filaments that are 10 nm long and attach to the cytosolic nuclear and

cellular membrane. Along with other proteins such as actin and microtubules, the keratins form the cytoskeleton of many epithelial cells, and staining with antibodies show that K18 is distributed in mammary cells, hepatocytes, and epidermal cells, among others. There is increased expression of K18 in many carcinomas including breast cancer, transitional cell carcinoma, hepatocellular carcinoma, pancreatic adenocarcinoma, colon adenocarcinoma and prostate cancer. However, nonepithelial cancer can also have aberrant K18 expression such as in the case of melanoma, and lymphoma.

The normal function of intracellular simple epithelial keratins may be to provide mechanical strength to the cells, but there may also be less well-defined functions. K18 may be involved in signal transduction through interactions with protein kinase C, or through interactions with the desmosome. There is some evidence that K18 is involved in apoptosis as a target of caspases when activated by other apoptotic stimuli and in supporting resistance to FAS-mediated apoptosis in hepatocytes.

The following table underscores the distribution of K18 in various cancers.

Table 1: K18 Distribution in Cancer

Cancer Type	N	Incidence	Source
Warthins tumor	26	100.0	Schwerer, Histopathology: 347, 2001
Wilms tumor	9	88.9	Rebhandl, Med Pediatr Oncol: 357, 2001
Squamous cell carcinomas of the oesophagus	35	97.0	Lam, Virchows Arch: 345, 1995
Poorly differentiated thyroid carcinoma	153	60.0	Lam, Eur J Surg Oncol: 631, 2001
Anaplastic thyroid carcinoma	153	80.0	Lam, Eur J Surg Oncol: 631, 2001
Medullary thyroid carcinoma	153	85.0	Lam, Eur J Surg Oncol: 631, 2001
Breast: medullary carcinoma	31	100.0	Lam, Eur J Surg Oncol: 631, 2001
Cholangiocarcinoma	77 min	77.0	Shimonishi, Histopathology: 55, 2000
Epithelioid hemangioendotheliomas	137 min	100.0	Miettinen, Hum Pathol: 1062, 2000
Epithelioid angiosarcomas	137 min	50.0	Miettinen, Hum Pathol: 1062, 2000
Angiosarcomas	137	20.0	Miettinen, Hum Pathol: 1062, 2000
Synovial sarcoma	110	100.0	Miettinen, Virchows Arch: 275, 2000
Pancreatic carcinoma	48	8.3	Thorban, Ann Oncol: 111, 1999
Squamous cell carcinoma	26		Depondt, Eur J Oral Sci: 442, 1999
Hepatocellular carcinoma	30	100.0	Tsuji, Pathol Int: 310, 1999
Cholangiocarcinoma	10	100.0	Tsuji, Pathol Int: 310, 1999
Infiltrating ductal breast carcinoma	101	majority	Malzahn, Virchows Arch: 119, 1998
Invasive ductal breast carcinoma	100	100.0	Rejthar, Neoplasma: 370, 1997
Hepatocellular carcinoma	20	55.0	D'Errico, Hum Pathol: 599, 1996
Cholangiocarcinoma	15	80.0	D'Errico, Hum Pathol: 599, 1996
Prostate carcinoma	84	33.0	Oberneder, Urol Res: 3, 1994
Prostate carcinoma	13	38.5	Riesenberg, Histochemistry: 61, 1993
Breast or gastrointestinal adenocarcinoma	532	33.0	Pantel, J Natl Cancer Inst: 1419, 1993
Melanoma	52	48.1	Fuchs, Am J Pathol: 169, 1992
Renal cell carcinoma	30	100.0	Dierick, Histopathology: 315, 1991
Hepatocellular carcinoma	34	100.0	Van Eyken, Hum Pathol: 562, 1988

There is also evidence for the extracellular expression of keratins. For example K18 has been identified as a hepatic receptor for thrombin-anti-thrombin complexes, and K8 on hepatocytes has been shown to bind plasminogen and tissue plasminogen activator. There is speculation that the extracellular expression of keratins may contribute to increased invasiveness in cancer.

Although K18 is well defined as a cancer marker, its role in the oncogenesis is less certain. Transfection of K8/K18 into melanoma increased their metastatic potential and their invasiveness, but this was not universal for all cancer types. Epithelium K8/K18 may provide resistance to Fas-mediated apoptosis and studies have found that the expression of K8 and K18 confer multiple drug resistance.

ARH460-23 is a monoclonal antibody that targets K18 and is being developed for therapeutic use in cancer. The commercial attractiveness of the antibody is underscored by the high percentage of cancers that express the H460-23 antigen. The predominant expression of the antigen on the cell surface of cancers and not on normal cells suggests the biology of the antigen is specifically drugable.

Biochemical Identification of the H460-23 Antigen

Identification of the H460-23 antigen involved two-dimensional electrophoresis and Western blotting (See Figs. 3 and 4). Membranes were prepared from ARH460-23 high (NCI H460) and ARH460-23 low (Jurkat) cell lines and analyzed with 2-D polyacrylamide gel electrophoresis to separate proteins according to their molecular weight and pH. The goal was to isolate a spot from the NCI H460 membrane proteins that was uniquely reactive with ARH460-23. Antibody 11E10 was used as a control.

ARH460-23 uniquely identified one protein spot from the NCI H460 membrane proteins. The corresponding protein to the one recognized on the Western blot was identified on the Sypro-stained gel. The spot had a molecular weight of 47.3 kDa and pI of 5.3. The protein was robotically spotted, excised from the gel, and digested with trypsin for matrix-assisted laser desorption/ionization (MALDI)/ mass spectrometry (MS) analysis. The MALDI/MS data were submitted to Profound (Proteometrics software package) for peptide mass fingerprint searching. Twenty-six of the 45

peptides generated from the spot matched cytokeratin 18. The peptides generated had 65% minimum sequence coverage of cytokeratin 18. These results indicate that cytokeratin 18 is likely the putative H460-23 antigen.

ARH60-23 Binding to Human Cancer Cell Lines

ARH460-23 binding was evaluated in 21 human cancer cell lines using flow cytometry to explore the distribution of the ARH460-23 target antigen across a range of human cancers. Antibodies of the same isotype, 11E10 was used as a negative control and antibody EOS9.1 which recognizes the CD95 antigen was used to probe whether the ARH460 target is CD95.

Figure 5 shows histogram profiles, and Table 2 summarizes H460-23 staining relative to the controls. The lung cell line, NCI H460, and umbilical vein epithelial cells, HUV-EC-C, show a high level of ARH460-23 expression compared to the other cell lines in this group. Overall, the majority of the solid tumors tested showed moderate levels of H460-23 antigen expression while the hematologic cancers, leukemia Jurkat and K562 and the T cell lymphoma K3P exhibited only marginal levels. The large cell lung carcinoma cell line NCI H661, unlike the NCI H460 cells, exhibited weak H460-23 staining. These findings suggest that ARH460-23 recognizes an antigen that is distributed differently on different types of cancer.

TABLE 2 Flow Cytometric Analysis of Cell Lines Stained with ARH460-23

Cell Line	Description	H460-23 Staining	EOS9.1 Staining
NCI H460	Large Cell Lung Cancer	Very Strong	Positive
Jurkat	Acute T cell Leukemia	Weak	Positive
K562	Chronic Myelogenous Leukemia	Weak	Negative
K3P	T cell Lymphoma	Weak	Positive
NCI H661	Large Cell Lung Cancer	Weak	Negative
HEY	Ovarian Epithelial Cancer	Strong	Positive
AU565	Mammary adenocarcinoma	Medium	Positive
T-47D	Mammary Ductal Carcinoma	Strong	Positive
MCF-7	Mammary Adenocarcinoma	Strong	Negative
EBV-transfected lymphocytes patient # 14725	Epstein Barr virus-in vitro transfected cells	Weak-Medium	Positive
EBV-transfected lymphocytes patient # 14935	Epstein Barr virus-in vitro transfected cells	Weak	Positive
HUV-EC-C	Human umbilical vein endothelial cells	Very Strong	Positive
A2058	Melanoma	Weak-Medium	Positive
Hs574.t	Mammary ductal carcinoma	Weak -Medium	Positive
Hs.888 Lu	Normal lung	Medium	N.D.
Hs574.Mg	Normal mammary	Medium	N.D.
SK-BR3	Mammary adenocarcinoma	Medium	Positive
CCD.27sK	Normal skin	Medium	N.D.
NCI-N87	Gastric carcinoma	Medium	Positive
BT-549	Mammary epithelial cancer	Medium	Positive
BxPC-8	Pancreatic adenocarcinoma	Strong	Positive

Target Validation

An immunohistochemistry study was carried out on formalin-fixed, paraffin-embedded human tissues by Qualtek Molecular Laboratories of Santa Barabara, CA to profile expression of the ARH460-23 antigen on normal and tumor tissues, and is summarized in Figure 7. Normal mouse serum was run in parallel as a negative control. Scoring of the specific antibody reactivity was done on the standard pathology scale of 0 to 4. Whenever possible the tissue was scored on a subcellular level to indicate reactivity within the nucleus, cytoplasm and plasma membrane.

Most tumor tissues tested were strongly positive for ARH460-23. In the colon and prostate all tumors were highly reactive and found to have both membranous and cytoplasmic binding. The one ovarian carcinoma tested yielded a similar result.

Lung and breast carcinomas and melanomas yielded more mixed results. In the lung, both large cell carcinomas were highly reactive on the cell membrane and in the cytoplasm. Of the 2 adenocarcinomas examined, one was positive for ARH460-23 and one negative. In breast, 7 of 9 carcinomas were positive. In medulary carcinomas where lymphocytes were present, the lymphocytes were also positive for ARH460-23. Two of the 4 melanomas tested were positive and reactive in both the cell membrane and cytoplasm.

In normal breast, skin, lung and ovary epithelia, ARH460-23 was either positive or weakly positive but confined to the cytoplasm and perinuclear regions of the cell. In both colon and ovary the normal tissues tested were negative for ARH460-23. However, normal colon displayed inflammatory cell reactivity in the stroma that was both membranous and cytoplasmic. When normal tissues were positive it was mainly confined to the cytoplasm and

perinuclear membranes. Typically when a tumor was positive for ARH460-23 it was found to be both reactive in the membrane and cytoplasm (16 of 18 or 89%). In addition, inflammatory cells found in proximity to tumor were reactive to ARH460-23 on the cell membrane and in the cytoplasm.

It was concluded that the antibody shows reactivity in both normal and neoplastic cells as well as populations of inflammatory cells in the colon and in proximity to tumors. The antibody tends to be reactive to cell membranes in many tumors and in lymphocytes but in normal epithelia it is mainly confined to cytoplasm and perinuclear regions.

All patents and publications mentioned in this specification are indicative of the levels of those skilled in the art to which the invention pertains. All patents and publications are herein incorporated by reference to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference.

It is to be understood that while a certain form of the invention is illustrated, it is not to be limited to the specific form or arrangement of parts herein described and shown. It will be apparent to those skilled in the art that various changes may be made without departing from the scope of the invention and the invention is not to be considered limited to what is shown and described in the specification. One skilled in the art will readily appreciate that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent therein. Any oligonucleotides, peptides, polypeptides, biologically related compounds, methods, procedures and techniques described herein are presently representative of the preferred embodiments, are intended to be exemplary and

are not intended as limitations on the scope. Changes therein and other uses will occur to those skilled in the art which are encompassed within the spirit of the invention and are defined by the scope of the appended claims. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are obvious to those skilled in the art are intended to be within the scope of the following claims.

CLAIMS

What is claimed is:

Claim 1. A method of treating a human tumor in a mammal, wherein said tumor expresses an antigen which specifically binds to a monoclonal antibody or antigen binding fragment thereof which has the identifying characteristics of a monoclonal antibody encoded by a clone deposited with the ATCC as accession number PTA-2700 comprising administering to said mammal said monoclonal antibody in an amount effective to reduce said mammal's tumor burden.

Claim 2. The method of claim 1 wherein said antibody is conjugated to a cytotoxic moiety.

Claim 3. The method of claim 2 wherein said cytotoxic moiety is a radioactive isotope.

Claim 4. The method of claim 1 wherein said antibody activates complement.

Claim 5. The method of claim 1 wherein said antibody mediates antibody dependent cellular cytotoxicity.

Claim 6. The method of claim 1 wherein said antibody is a murine antibody.

Claim 7. The method of claim 1 wherein said antibody is a humanized antibody

Claim 8. The method of claim 1 wherein said antibody is a chimerized antibody.

Claim 9. An isolated monoclonal antibody or antigen binding fragments thereof encoded by the clone deposited with the ATCC as PTA-2700.

Claim 10. The isolated antibody or antigen binding fragments of claim 9, wherein said isolated antibody or antigen binding fragments thereof is humanized.

Claim 11. . The isolated antibody or antigen binding fragments of claim 9 conjugated with a member selected from the group consisting of cytotoxic moieties, enzymes, radioactive compounds, and hematogenous cells.

Claim 12. The isolated antibody or antigen binding fragments of claim 9, wherein said isolated antibody or antigen binding fragments thereof is a chimerized antibody.

Claim 13. The isolated antibody or antigen binding fragments of claim 9, wherein said isolated antibody or antigen binding fragments thereof is a murine antibody.

Claim 14. The isolated clone deposited with the ATCC as PTA-2700.

Claim 15. A binding assay to determine presence of cancerous cells in a tissue sample selected from a human tumor comprising:

providing a tissue sample from said human tumor ;

providing an isolated monoclonal antibody or antigen binding fragment thereof encoded by the clone deposited with the ATCC as PTA-2700;

contacting said isolated monoclonal antibody or antigen binding fragment thereof with said tissue sample; and

determining binding of said isolated monoclonal antibody or antigen binding fragment thereof with said tissue sample;

whereby the presence of said cancerous cells in said tissue sample is indicated.

Claim 16. The binding assay of claim 15 wherein the human tumor tissue sample is obtained from a tumor originating in a tissue selected from the group consisting of colon, ovarian, lung, and breast tissue.

Claim 17. A process of isolating or screening for cancerous cells in a tissue sample selected from a human tumor comprising:

providing a tissue sample from a said human tumor ;

providing an isolated monoclonal antibody or antigen binding fragment thereof encoded by the clone deposited with the ATCC as PTA-2700;

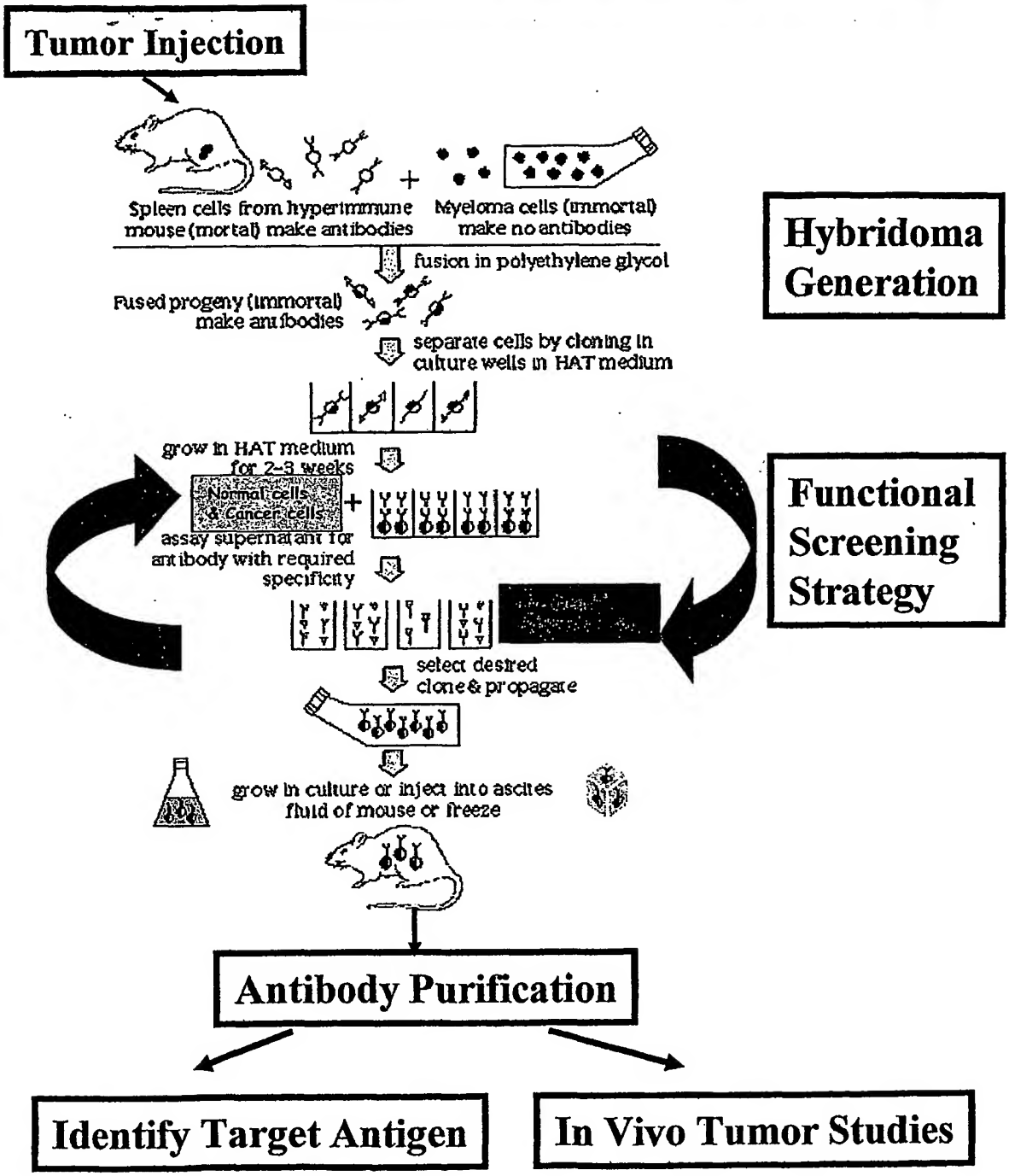
contacting said isolated monoclonal antibody or antigen binding fragment thereof with said tissue sample; and

determining binding of said isolated monoclonal antibody or antigen binding fragment thereof with said tissue sample;

whereby said cancerous cells are isolated by said binding and their presence in said tissue sample is confirmed.

Claim 18. The process of claim 17 wherein the human tumor tissue sample is obtained from a tumor originating in a tissue selected from the group consisting of colon, ovarian, lung, and breast tissue.

Strategy For Anti-Cancer Antibody Generation



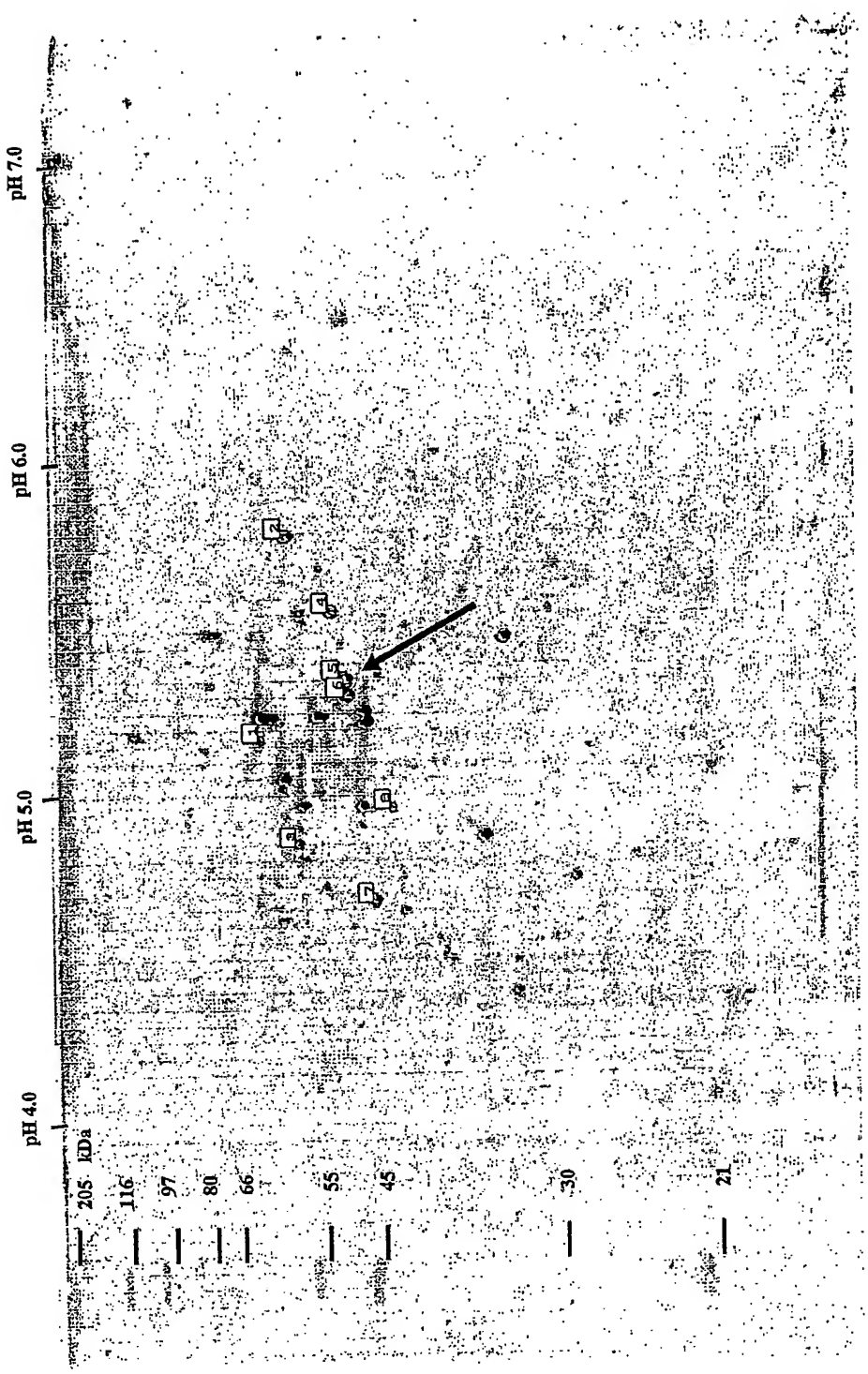
SUBSTITUTE SHEET (RULE 26)

ARH460-23 Alone Significantly Reduced Metastasis of Lung Tumor (NCI H460) In Orthotopic Implantation Model

Group	Mouse	Metastasis and sites			Final primary tumor weight (g)
		Contralateral lung	Thoracic LN		
Cisplatin 3.5 mg/kg	Positive	6/9	7/9	Mean	1.52
				SD	0.36
	P value vs. control	1	1		0.27
ARH460-23A 25 mg/kg	Positive	3/10	2/10	Mean	1.23
				SD	0.25
	P value vs. control	0.074	0.007		0.023
Cisplatin 3.5 mg/kg and ARH460-23A 25 mg/kg	Positive	5/9	4/9	Mean	1.38
				SD	0.55
	P value vs. control	0.650	0.170		0.084
Control	Positive	7/10	8/10	Mean	1.99
				SD	0.72

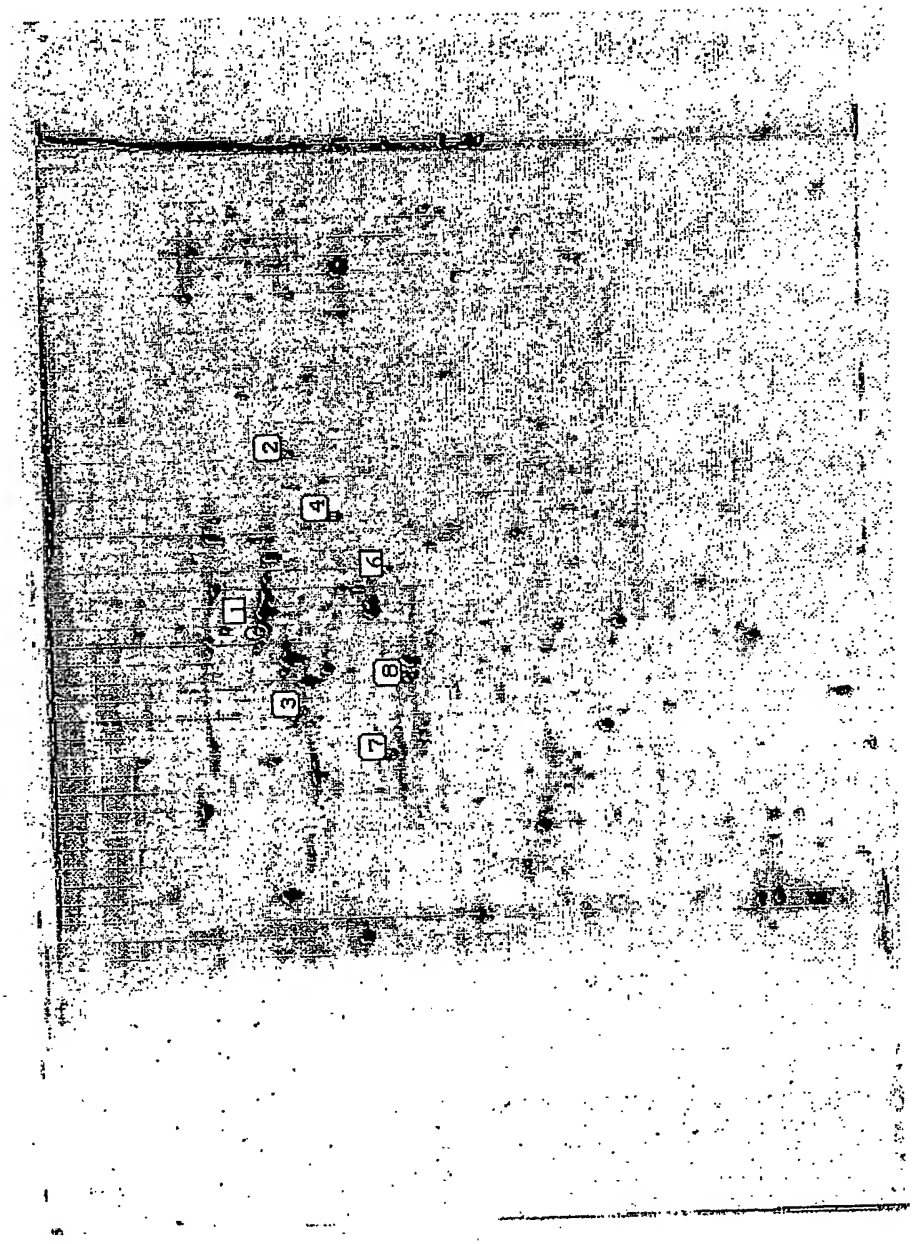
NCr-nu mice were treated with antibody and/or cisplatin following orthotopic implantation of GFP-labelled NCI H460 cells. Mice were given ARH460-23 at 3 days post implantation and cisplatin at 1, 5, and 9 days post implantation. Primary tumor weights were recorded and tumor metastasis to the contralateral lung and thoracic lymph nodes (LN) was determined at 42 days post implantation. This work was done in collaboration with Anti-Cancer Inc., San Diego, CA.

BEST AVAILABLE COPY



2-D gel analysis of NCI H460 membrane proteins probed with ARH460-23. Membrane lysates were run on 2-D gels and stained with sypro ruby (gel above) or transferred to a blot for probing with ARH460-23. Spot 5 (see arrow) was uniquely reactive to ARH460-23 as this spot was not detectable in a 2-D blot of Jurkat membrane proteins. By matrix-assisted laser desorption /ionization/mass spectrometry analysis, this spot was identified as human cytokeratin 18.

BEST AVAILABLE COPY



2-D gel analysis of membrane proteins from Jurkat cells probed with ARH460-23. Jurkat cell membrane lysates were run on 2-D gels and stained with sypro ruby (gel above) or transferred to a membrane blot and probed with ARH460-23. Spot 5 (cytokeratin 18) was not detected in the 2-D blot.

BEST AVAILABLE COPY

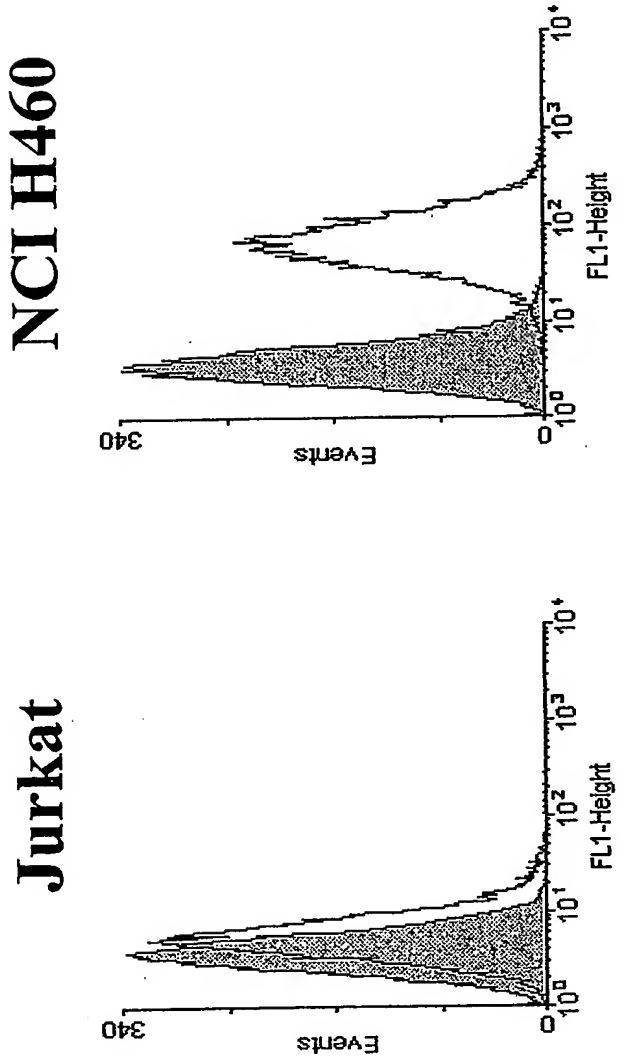
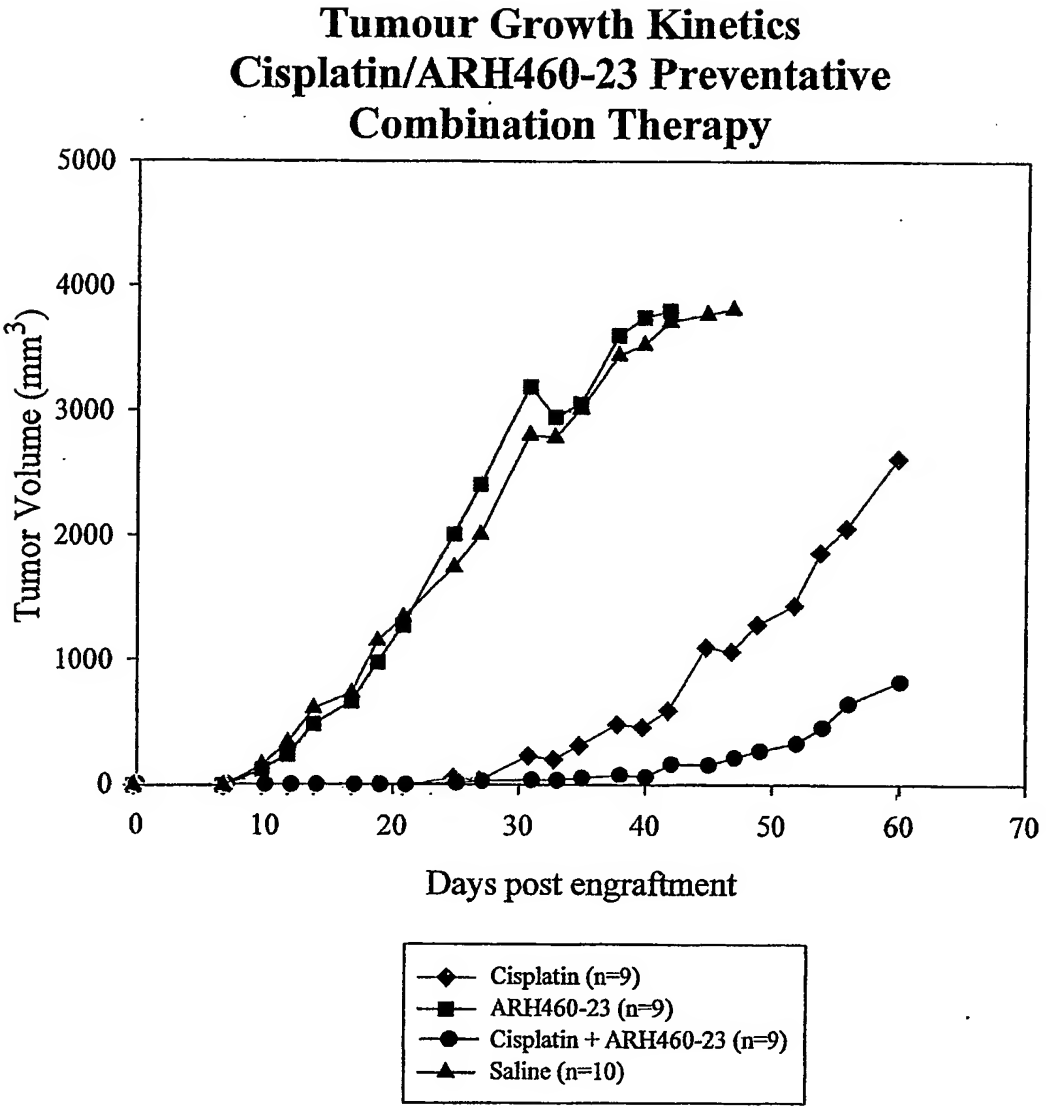


Figure. Differential reactivity of ARH460-23 for Jurkat and NCI H460 cells. Cells were stained with either 2 μg of purified ARH460-23 or the isotype control mouse IgM antibody 11E10 followed by Alexa Fluor 488 conjugated goat anti-mouse IgM. The histogram plots of isotype control staining (shaded) are compared with ARH460-23 staining (dark outline).



The effect of preventative therapy on tumour growth kinetics. Average tumor volumes are plotted as a function of time. Combination treatment of cisplatin and ARH460-23 significantly reduced the tumor growth rate ($p<0.05$).

Immunohistochemistry staining results with Arius H460-23 (QML Project # 419)

Results from 20 minute SHIER / no enzyme tissue pretreatment (overnight H460-23 incubation at 3.0µg/ml)

TISSUE TYPE	SAMPLE NO.	TISSUE PATHOLOGY	Reactivity	Cellular Stain location	Stain Scale 1-4	Mouse IgG Control	Comments
COLON	1	NORMAL epithial glands	NEGATIVE			NEGATIVE	
	2	CARCINOMA	POSITIVE	Membranous, perinuclear and cytoplasm	4	NEGATIVE	
		NORMAL ADJACENT	NEGATIVE				
	3	CARCINOMA	POSITIVE	Membranous, perinuclear and cytoplasm	4	NEGATIVE	Inflamatory cells also positive
		NORMAL ADJACENT	NEGATIVE				
PROSTATE	4	NORMAL	POSITIVE	Cytoplasm	4	NEGATIVE	
	5	CARCINOMA	POSITIVE	Membranous, perinuclear and cytoplasm	4	NEGATIVE	Inflamatory cells also positive
	6	CARCINOMA	POSITIVE	Membranous, perinuclear and cytoplasm	4	NEGATIVE	Inflamatory cells also positive
	7	CARCINOMA GLEASON GRADE 5	POSITIVE	Membranous, perinuclear and cytoplasm	4	NEGATIVE	Inflamatory cells also positive
LUNG	8	NORMAL	POSITIVE	Cytoplasm and perinuclear	4	NEGATIVE	Bronchial Columnar Epithelial cells negative
	9	ADENOCARCINOMA	NEGATIVE		2	NEGATIVE	Inflamatory cells also positive
	10	ADENOCARCINOMA	POSITIVE	Membranous and cytoplasm	4	NEGATIVE	Inflamatory cells also positive
	11	LARGE CELL CARCINOMA	POSITIVE	Membranous and cytoplasm	4	NEGATIVE	Inflamatory cells also positive
	12	LARGE CELL CARCINOMA	POSITIVE	Membranous and cytoplasm	4	NEGATIVE	Inflamatory cells also positive
OVARY	13	NORMAL	NEGATIVE			NEGATIVE	Stroma light
	14	CARCINOMA	POSITIVE	Membranous and cytoplasm	4	NEGATIVE	Stroma Positive
SKIN	15	NORMAL	POSITIVE	Cytoplasm and perinuclear	4	NEGATIVE	
	16	MELANOMA - Metastasis to breast	POSITIVE	Cytoplasm	4	NEGATIVE	Inflamatory cells also positive
	17	NODULAR MELANOMA	POSITIVE	Membranous and cytoplasm	4	NEGATIVE	Positive melanin Melanocytes
	18	MELANOMA - residual recurrent invasive melanoma	NEGATIVE			NEGATIVE	
	19	MELANOMA - trabecular with lymphatic involvement	NEGATIVE			NEGATIVE	
BREAST	20	NORMAL	WEAKLY POSITIVE	Cytoplasm	2	NEGATIVE	
	21	DUCTAL INVASIVE CARCINOMA Moderate to poorly differentiated	WEAKLY POSITIVE	Membranous and cytoplasm	3	NEGATIVE	
	22	DUCTAL INVASIVE CARCINOMA well to moderately differentiated	NEGATIVE			NEGATIVE	
	23	DUCTAL INVASIVE CARCINOMA moderately differentiated	NEGATIVE			NEGATIVE	
	24	DUCTAL INVASIVE CARCINOMA poorly differentiated	POSITIVE	Cytoplasm and perinuclear	4	NEGATIVE	
	25	MEDULARY CARCINOMA well differentiated	POSITIVE	Membranous and cytoplasm	3	NEGATIVE	Inflamatory cells also positive
	26	DUCTAL INVASIVE CARCINOMA well to moderately differentiated	NEGATIVE			NEGATIVE	
	27	DUCTAL INVASIVE CARCINOMA well to moderately differentiated	POSITIVE	Membranous and cytoplasm	4	NEGATIVE	
	28	NORMAL	WEAKLY POSITIVE	Cytoplasm	2	NEGATIVE	
	29	DUCTAL INVASIVE CARCINOMA well to moderately differentiated	POSITIVE	Membranous and cytoplasm	4	NEGATIVE	
	30	HYPERPLASIA	POSITIVE	Cytoplasm	4	NEGATIVE	
	31	MEDULARY CARCINOMA well differentiated	POSITIVE	Membranous and cytoplasm	4	NEGATIVE	Inflamatory cells also positive
CONTROL CELL PELLETS	32	NCI-H360 Cells	POSITIVE	Membranous and cytoplasm	4	NEGATIVE	Figure 1
	33	Jurkat Cells	NEGATIVE			NEGATIVE	Figure 1

BEST AVAILABLE COP

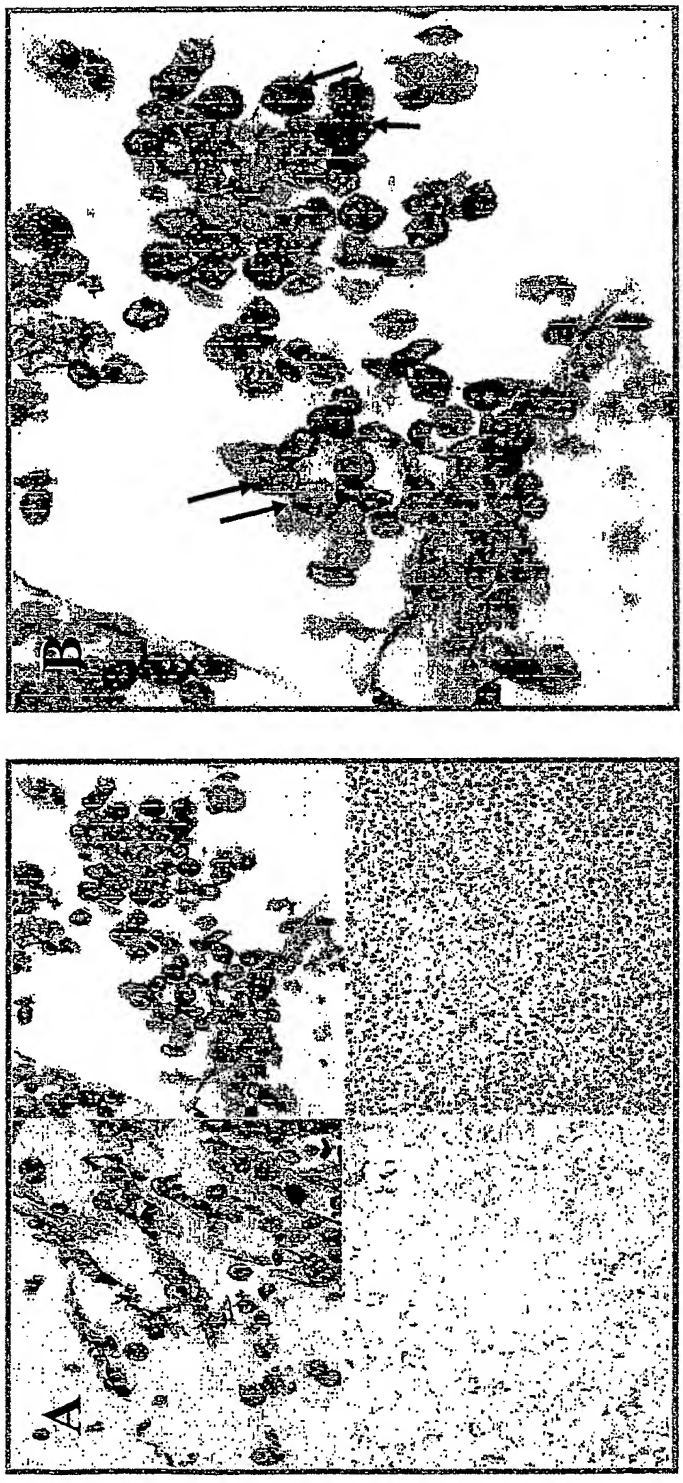


Figure A. Immunohistochemical staining of paraffin-embedded, formalin-fixed NCI H460 (top) and Jurkat (bottom) cell pellets with ARH460-23. ARH460-23 specifically reacted with NCI H460 cells.

Figure B. Immunohistochemical staining of paraffin-embedded, formalin-fixed NCI H460 (top) cell pellets with ARH460-23. ARH460-23 reacted with both the cell membrane (black arrows) and cytoplasm (blue arrows) of NCI H460 cells.

This Page is Inserted by IFW Indexing and Scanning Operations and is not part of the Official Record

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☑ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☐ FADED TEXT OR DRAWING
- ☐ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☑ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☑ GRAY SCALE DOCUMENTS
- ☐ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☐ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: _____

IMAGES ARE BEST AVAILABLE COPY.
As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.

THIS PAGE BLANK (USPTO)